Efficacy of the UK recombinant plague vaccine to protect against pneumonic plague in the nonhuman primate, *Macaca fascicularis*

{PRIVATE }

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Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The views, opinions and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

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14. ABSTRACT

The efficacy of the UK candidate plague vaccine was established in the cynomolgus macaque model for pneumonic plague. Animals were vaccinated intramuscularly on days 0 and 21 with either 40 µg F1 + 40 µg V or 80 µg F1 + 80 µg V in 0.5 ml 20% v/v Alhydrogel. They were challenged on day 60 with a lethal aerosol challenge (> 100 LD50) of CO 92 Y. pestis. All but one of the 19 vaccinated animals that were challenged survived.

15. SUBJECT TERMS

Yersinia pestis, plague, F1-V fusion protein vaccine, CpG oligodeoxynucleotide antigens, efficacy, antibody response, aerosol challenge, laboratory animals, nonhuman primates

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ABSTRACT

The efficacy of the UK candidate plague vaccine was established in the cynomolgus macaque model for pneumonic plague. Animals were vaccinated intramuscularly on days 0 and 21 with either 40 μg F1 + 40 μg V or 80 μg F1 + 80 μg V in 0.5 ml 20%v/v Alhydrogel. They were challenged on day 60 with a lethal aerosol challenge (>100 LD_{50}) of CO 92 Y. pestis. All but one of the 19 vaccinated animals that were challenged survived.

1 INTRODUCTION

Plague is an infection caused by the gram negative bacterium *Yersinia pestis*. Plague is an infection of small rodents and mammals - for example the rat. In the rodent population the disease exists in many forms covering a spectrum from acute to chronic illness.

Plague has been responsible for a number of human epidemics - most notably the Black Death in the Middle Ages [1]. Transmission from rodent to man usually relies on a flea vector - although cases of pulmonary transmission have been described from household pets. Transmission to man by feeding fleas leads to the characteristic swelling of the draining lymph nodes, followed by a septicaemic illness. This presentation is the classical bubonic plague [2]. However, man-to-man transmission can occur via droplet nuclei spread by the coughing of patients with bubonic or septicaemic plague who have developed pulmonary lesions.There is a need for a less reactogenic, more protective and simpler to produce vaccine than the killed whole cell vaccine that was formerly produced as the USP plague vaccine. A new sub-unit vaccine for plague has been researched and developed at DSTL, Porton Down in the UK and this vaccine is now entering manufacture. The vaccine comprises the F1 and V sub-unit antigens of *Yersinia pestis*, which have been produced as purified recombinant proteins, derived from an *E. coli* host. The vaccine is formulated by adsorbing the F1 and V antigens to alhydrogel.

The preclinical studies in mice have shown that the F1 + V sub-unit combination vaccine provides protection against an aerosolised challenge with *Y. pestis* [3-6]. Pharmaco-Toxicological Testing has been completed and shown

- the vaccine to be safe in nonhuman primates (NHP) pharmacology studies measuring cardiovascular/respiratory parameters
- the vaccine has no adverse effects on the CNS in studies in mice.
- the vaccine has no toxic effects by acute toxicity, repeat dose testing, local tolerance testing & reproductive toxicity testing

A Phase I Clinical Trial has demonstrated safety in a small number of human volunteers as required by the regulatory authorities.

Normal Phase III studies are not possible when developing vaccine as a countermeasure against plague since, there is low risk of disease in the normal population and it would be unethical to challenge humans with live micro-organisms. Therefore there is a need to establish efficacy through the use of surrogate markers of protection as described in the FDA new rule (21 CFR Parts 314 and 601) and EMEA Guidance - CPMP/EWP/463/97. In essence animal models must be used as a means of establishing a relationship between protection in animals and humans. In light of this, Passive Transfer and a number of surrogate markers have been assessed in the Phase I Clinical Trial, as possible indicators of protection in Man.

The above plus the need to give confidence to the customer that the plague vaccine will protect a Non-Human Primate and is thus likely to protect man mean that there is a requirement to assess the efficacy of the UK Recombinant Plague Vaccine in a NHP.

To date several studies have been completed in the cynomolgus macaque which have demonstrated the F1+V vaccine to be immunogenic in the dose range of 5-40μg each of F1+V adsorbed to alhydrogel and schedule (day 0,21) intended for use in man. Passive transfer of sera or of IgG fractionated from the sera of vaccinated macaques into the

naïve mouse with subsequent challenge of the mouse has been carried out. This has shown that sera from vaccinated macaques are consistently protective against challenge from approximately day 28 of the immunization schedule through to week 53 after two immunizing doses of vaccine.

Further, sera from the vaccinated macaques have been demonstrated to compete with a neutralizing monoclonal antibody for binding to the V antigen, in a competitive ELISA. The competitive binding data correlate with the passive transfer data for the macaque sera. Such data provide surrogate markers of efficacy for the F1+V vaccine in the cynomolgus macaque and the same set of assays for surrogate markers of efficacy have been applied to blood samples from individuals in Phase 1 clinical trials of the vaccine.

In this study, cynomolgus macaques were vaccinated with formulated F1 + V vaccine using the same dose as in the Phase I clinical trial (40 μg each of F1 and V) and a higher dose of 80 μg each of F1 and V. The vaccination schedule (days 0 and 21) was the same as that used in the Phase I clinical trial. On day 60 the animals were challenged with a lethal aerosol challenge of *Y. pestis*.

2. MATERIALS AND METHODS

2.1 Experimental animals

Cynomolgus macaques, males and females, 3 – 8 kg, were obtained from USAMRIID colony.

2.2 Telemetry Transducer Implants:

Telemetry devices to measure temperature and activity (TA10TA-D70) were implanted subcutaneously in all cynomolgus macaques approximately 30 days prior to vaccination. These transducers were placed subcutaneously on the dorsum of the animal between the scapula, just off the midline at the level of the inferior edge of the scapular blade.

2.3 Vaccine

Formulated vaccine containing 120 μg rF1 and 120 μg rV was received from Octoplus – Holland. Placebo (diluent) was received from Baxter.

2.3.1 Vaccine Information

120/120 Microgram/0.5ml rV/rF1 containing 20% alhydrogel in saline.

Batch Number 03D11601-04A

Store at 5+/-3^{o}C.

Retest Date 18/10/2003

Manufactured for DSTL

2.3.2 Diluent

Batch # 803634

PC # 262-101-100

Client

Date 8/8/03

Temp $2^{o}C$-$8^{o}C$

2.4 Preparation of Vaccine

The vaccine was diluted to obtain the required vaccines dosages of 40/40µg and 80/80µg according to the following:

22 vials of formulated120/120 µg were mixed, opened and pooled to give approximately 12ml of vaccine suspension.

40/40 µg

4ml of vaccine + 8ml of Diluent.

(volumes dispensed using a graduated pipette)

80/80 µg

7.5ml of vaccine + 3.75ml of Diluent.

(large volumes dispensed using a graduated pipette. Volumes under 1ml dispensed using a micropipette)

Prepared vaccine dilutions were kept on ice throughout their period of use.

4 x 1ml volumes of each vaccine was aliquoted for shipment to Cylex. At Cylex they were assayed to determine the actual concentration of protein (data not available to USAMRIID)

2.5 Vaccination and Challenge Schedule

Twenty-two Cynomolgus macaques (*M. fascicularis*) were divided into two groups of ten and one group of two. The 2 groups of 10 animals were vaccinated on days 0, and 21 with either 40 μg F1 + 40 μg V or 80 μg F1 + 80 μg V in 0.5 ml 20%v/v Alhydrogel, while the group of 2 received placebo, Saline/Alhydrogel, according to the same schedule. The animals were bled immediately prior to vaccination, and then on days 7, 14, 21, 28, 35, 42, 50 (10 ml) and 59 (20 ml). The blood was used to determine serum antibody levels to both F1 and V and assess cell mediated immunity. The day 59 sera will be used for passive transfer, IgG levels to F1 and V, competitive ELISAs and cell cytoxicity assays, and cell mediated immunity (data not available to USAMRIID).

One vaccinated animal died prior to challenge of unrelated causes. The primary cause of death was determined to be acute cardiac decompensation secondary to chronic heart disease. The rest of the vaccinated animals were randomly divided into 2 challenge groups, with one control in each group, and were challenged on 2 consecutive days with approximately 126 ± 36 LD_{50} of the F1 positive *Y. pestis* strain, CO92.

2.6 *Y. pestis* challenge

Two days before the aerosol challenge, Tryptose blood agar base (TBAB) culture slants were inoculated with *Y. pestis*, Colorado 92. These were incubated for two days at 26 – 30 ^{o}C. On the day of challenge, each slant culture was suspended in 1-2 ml of Heart Infusion Broth (HIB). Suspensions from the slants were pooled, vortexed for 10 sec. and the optical density (O.D.) of the suspension determined at 620 nm. (1 unit of optical density equates to a *Y. pestis* concentration of 10^9 cfu/ml.).

The nonhuman primates were anesthetized with Telazol (6 mg/kg IM) for the aerosol challenge. Respiratory minute volumes were measured by whole body plethysmography using a Buxco Biosystem XA (Buxco Electronics, Sharon, CT), immediately before challenge. The animals were then immediately exposed to the bacterial aerosol, head-only, in a dynamic aerosol chamber controlled using the Automated Bioaerosol Exposure System. The aerosol (mass median aerosol diameter, 1.2 μm) was generated by a three-jet Collison nebulizer and sampled continuously by an all-glass impinger (AGI-30; Ace Glass, Inc., Vineland, N.J.). For each animal, the aerosol concentration of *Y. pestis* organisms was calculated by plating out dilutions of a sample from the AGI onto blood agar plates (Remel). The inhaled doses were then determined (expressed as LD_{50}). One aerosol LD_{50} in the cynomolgus macaque is 400 organisms (MLM Pitt, unpublished observation).

2.7 Temperature Monitoring

Temperature data was continuously collected hourly for one week prior to challenge and then for 14 days post challenge using Dataquest A.R.T. 2.3 software.

3. RESULTS

Eight of the nine animals that were vaccinated with 40 µg F1 + 40 µg V in 0.5 ml 20%v/v Alhydrogel survived challenge. All 10 of the nonhuman primates that were vaccinated with 80 µg F1 + 80 µg V in 0.5 ml 20%v/v Alhydrogel survived challenge. The 2 controls (49470 and 99310) succumbed to pneumonic plague; one was euthanized 89 hr post exposure and the other died 86 hr post exposure (Figure 1 and 2). Both were bacteremic in the 24 hr prior to death.

The vaccinated animal that died, developed a fever around 73 hr post-challenge, became bacteremic by 90 hr and died around 128 hr post-exposure. (Figure 3)

Figure 1:

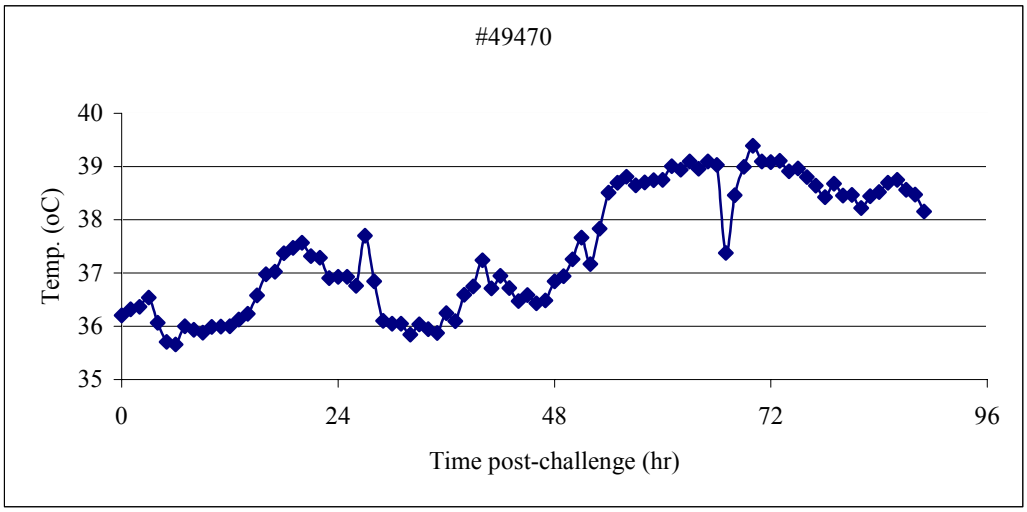


Figure 2:

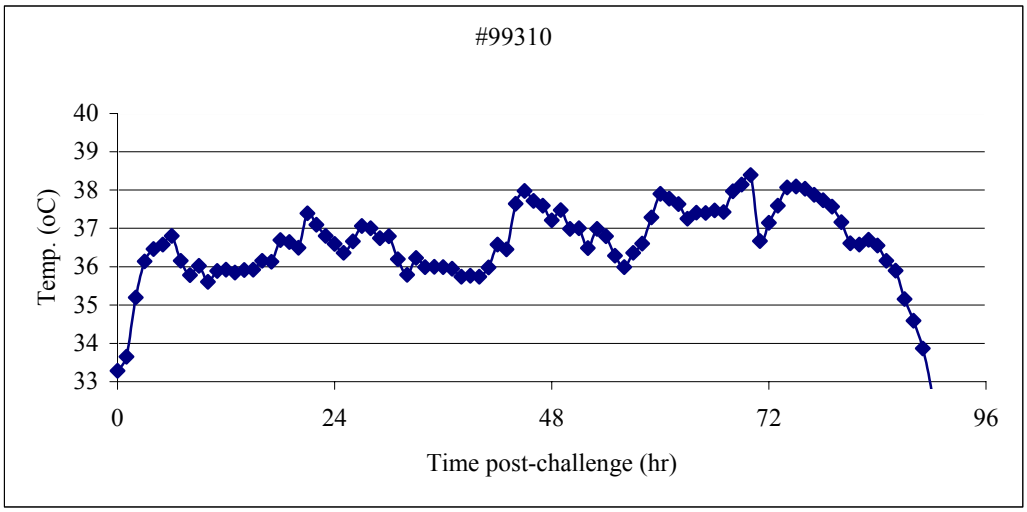
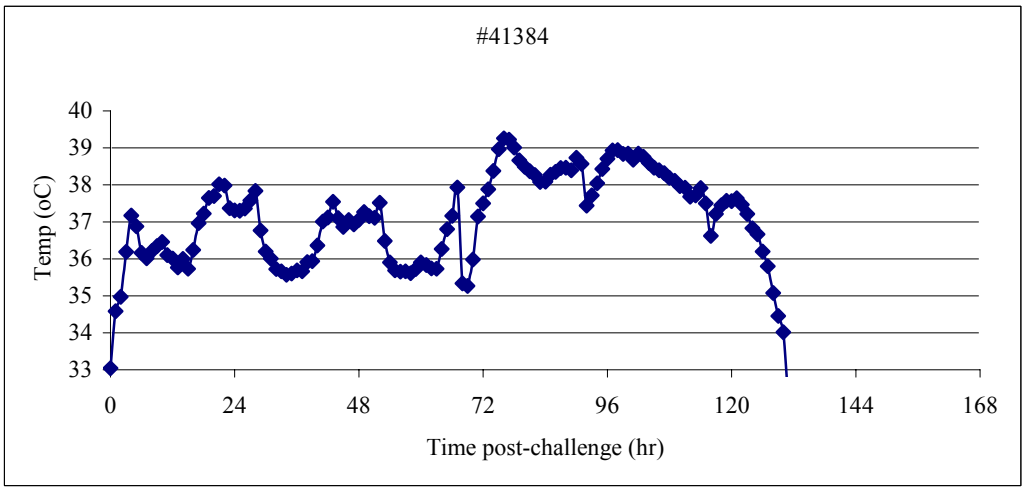


Figure 3:



MCMR-UIZ-L (70-1n) 27 June 2003

MEMORANDUM THRU Chief, Toxinology & Aerobiology Division

FOR Dr. M. Louise Pitt, Toxinology & Aerobiology Division

SUBJECT: Protocol Approval

1. Your protocol (copy enclosed) entitled "Efficacy of the UK Recombinant Plague Vaccine to Protect Against Pneumonic Plague in the Nonhuman Primate, *Macaca fasicularis*" was approved by the LACUC and MRMC and assigned protocol number F03-11. The approval date of this protocol is 23 June 2003. Please use this protocol number when ordering animals, submitting samples to the clinical laboratory, submitting samples for pathological evaluation, etc.

2. This protocol will be reviewed by the LACUC annually (or sooner if the estimated completion date is less than a year) for animal use issues in compliance with federal law. Meanwhile, please advise the LACUC Chair if there are any changes in personnel, the experimental design, the pain status, or a significant change in the number of animals authorized so that the protocol record may be properly annotated.

3. Please notify LTC Carol Eisenhauer at X34705, when this protocol has been completed so the animal account can be closed and the "Report of Protocol Completion/Termination" form can be forwarded to the P.I. for completion and return to the LACUC.

Carol Eisenhauer

Encl

CAROL L. EISENHAUER
LTC, VC
LACUC Chair

USAMRIID RESEARCH PROTOCOL

Proposal No. 03-34 Protocol No F03-11

Date Approved: 23 Jun 03

APC: Research Plan: 02-4-AA-003

Title: Efficacy of the UK recombinant plague vaccine to protect against pneumonic plague in the nonhuman primate, *Macaca fascicularis*

Principal Investigator/Division/Phone #: M. Louise M. Pitt Date 7 May 03
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EXT: 34230 M. LOUISE M. PITT

Division Chief Review and Approval: Date 13 MAY 03
(Sign and Print Name)
MAJ CB MILLARD 3-4261

Research Area Coordinator: Date 7 May 03
(Sign and Print Name) Patricia Worsham

Attending/Consulting Veterinarian: Julie Stephens Date 13 May 03
(Sign and Print Name)

Statistical Review (Biostatistican) Date 07 MAY 2003
(Sign and Print Name) Paul Gibbs

Safety Office Review: Robert J. Hawley Date 7 MAY 03
Robert J. Hawley
(Sign and Print Name)

LACUC CHAIR:
Recommend Approval:
Disapproved: Date 20 JUNE 03
Susan D. Goodwin MAJ, VC
(Sign and Print Name)

USAMRIID Commander:
Approved: Date 6/23/03
(Sign and Print Name)

PROTOCOL NUMBER:

PROTOCOL TITLE: Efficacy of the UK recombinant plague vaccine to protect against pneumonic plague in the nonhuman primate, *Macaca fascicularis*

PRINCIPAL INVESTIGATOR\DIVISION: M. Louise M. Pitt, Ph.D., Toxinology & Aerobiology.

CO-INVESTIGATOR(S)\DIVISION(S):

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Mr. D. Dyer, Toxinology & Aerobiology
MAJ M. Tate, Veterinary Medicine
MAJ P. Rico, Veterinary Medicine
CPT S. Gamble, Veterinary Medicine
Mr. Keith Esham, Veterinary Medicine
Dr. G. Andrews, Bacteriology

I. NON-TECHNICAL SYNOPSIS:

In this protocol, we will determine if the UK recombinant plague vaccine, a mixture of 2 recombinant antigens F1 and V combined with an adjuvant containing aluminum, will protect cynomolgus monkeys from pneumonic plague.

II. A. BACKGROUND:

Plague is an infection caused by the gram negative bacterium *Yersinia pestis*. Plague is an infection of small rodents and mammals - for example the rat. In the rodent population the disease exists in many forms covering a spectrum from acute to chronic illness.

Plague has been responsible for a number of human epidemics - most notably the Black Death in the Middle Ages [1]. Transmission from rodent to man usually relies on a flea vector - although cases of pulmonary transmission have been described from household pets. Transmission to man by feeding fleas leads to the characteristic swelling of the draining lymph nodes, followed by a septicaemic illness. This presentation is the classical bubonic plague [2]. However, man-to-man transmission can occur via droplet nuclei spread by the coughing of patients with bubonic or septicaemic plague who have developed pulmonary lesions. There is a need for a less reactogenic, more protective and simpler to produce vaccine than the killed whole cell vaccine that was formerly produced as the USP plague vaccine. A new sub-unit vaccine for plague has been researched and developed at DSTL, Porton Down in the UK and this vaccine is now entering manufacture. The vaccine comprises the F1 and V sub-unit antigens of *Yersinia pestis*, which have been produced as purified recombinant proteins, derived from an *E. coli* host. The vaccine is formulated by adsorbing the F1 and V antigens to alhydrogel.

The preclinical studies in mice have shown that the F1 + V sub-unit combination vaccine provides protection against an aerosolised challenge with *Y. pestis* [3-6].
Pharmaco-Toxicological Testing has been completed and shown:

- the vaccine to be safe in nonhuman primates (NHP) pharmacology studies measuring cardiovascular/respiratory parameters
- the vaccine has no adverse effects on the CNS in studies in mice.
- that the vaccine has no toxic effects by acute toxicity, repeat dose testing, local tolerance testing & reproductive toxicity testing

A Phase I Clinical Trial has demonstrated safety in a small number of human volunteers as required by the regulatory authorities.

Normal Phase III studies are not possible when developing vaccine as a countermeasure against plague since, there is low risk of disease in the normal population and it would be unethical to challenge humans with live micro-organisms. Therefore there is a need to establish efficacy through the use of surrogate markers of protection as described in the FDA new rule (21 CFR Parts 314 and 601) and EMEA Guidance - CPMP/EWP/463/97. In essence animal models must be used as a means of establishing a relationship between protection in animals and humans. In light of this, Passive Transfer and a number of surrogate markers have been assessed in the Phase I Clinical Trial, as possible indicators of protection in Man.

The above plus the need to give confidence to the customer that the plague vaccine will protect a Non-Human Primate and is thus likely to protect man mean that there is a requirement to assess the efficacy of the UK Recombinant Plague Vaccine in a NHP.

To date several studies have been completed in the cynomolgus macaque which have demonstrated the F1+V vaccine to be immunogenic in the dose range of 5-40μg each of F1+V adsorbed to alhydrogel and schedule (day 0,21) intended for use in man. Passive transfer of sera or of IgG fractionated from the sera of vaccinated macaques into the naïve mouse with subsequent challenge of the mouse has been carried out. This has shown that sera from vaccinated macaques are consistently protective against challenge from approximately day 28 of the immunization schedule through to week 53 after two immunizing doses of vaccine.

Further, sera from the vaccinated macaques have been demonstrated to compete with a neutralizing monoclonal antibody for binding to the V antigen, in a competitive ELISA. The competitive binding data correlate with the passive transfer data for the macaque sera. Such data provide surrogate markers of efficacy for the F1+V vaccine in the cynomolgus macaque and the same set of assays for surrogate markers of efficacy have been applied to blood samples from individuals in Phase 1 clinical trials of the vaccine.

In this study, cynomolgus macaques will be vaccinated with formulated F1 + V vaccine using the same dose as in the Phase I clinical trial (40 μg each of F1 and V) and a higher dose of 80 μg each of F1 and V. The vaccination schedule (days 0 and 21) will be the same as that used in the Phase I clinical trial. On day 60 the animals will then be challenged with a lethal aerosol challenge of *Y. pestis*.

B. Literature Search:

1. Literature Sources Searched: Medline, FEDRIP, BIOSIS, DTIC, BRD
2. Date and Number of Search: 1565, 29 May 2003
3. Key Words of Search: Pneumonic plague, vaccine, aerosol, F1 antigen, V antigen, African Green monkey, cynomolgus monkeys.

4. Results of Search: There were references found to F1 based vaccines and a rF1-V vaccine which have been shown to be efficacious against either parenteral or aerosol challenge in mice. There were no references found for a F1 +V combination vaccine tested in a cynomolgus monkey against an aerosol challenge of live plague bacilli. This study does not duplicate any previous work.

III. OBJECTIVE\HYPOTHESIS:

1. To determine whether the UK candidate vaccine consisting of recombinant F1and V antigens combined with Alhydrogel can protect against a lethal aerosol challenge of *Y. pestis* in *M. fascicularis*.

IV. MILITARY RELEVANCE:

Y. pestis is both an infectious disease threat and a biological warfare threat to U.S. military personnel. It is therefore imperative that a vaccine that protects against both bubonic and pneumonic plague is available.
DTO: CB 34

V. MATERIALS AND METHODS:

A. Experimental Design and General Procedures:

1. Twenty-two Cynomolgus macaques (*M. fascicularis*) will be divided into two groups of ten and one group of two.
The 2 groups of 10 animals will be vaccinated on days 0, and 21 with either 40 μg F1 + 40 μg V or 80 μg F1 + 80 μg V in 0.5 ml 20%v/v Alhydrogel (Table 1), while the group of 2 will receive placebo, Saline/Alhydrogel, according to the same schedule. On day 60 all the animals will be challenged with approximately 100 LD_{50} of the F1 positive *Y. pestis* strain, CO92.

TABLE 1

Group	#/group	Treatment	Challenge
1	10	40 μg F1 + 40 μg V	100 LD_{50} CO92
2	10	80 μg F1 + 80 μg V	100 LD_{50} CO92
3	2	Placebo	100 LD_{50} CO92

3. Telemetry devices to measure temperature and activity will be implanted subcutaneously in all cynomolgus macaques approximately 30 days prior to vaccination. If possible, animals that already have telemetry device implanted will be used.

4. Telazol (2 mg/kg IM) or Ketamine/acepromazine mixture @ 0.1 ml/kg (9 mg/kg ketamine and 0.1 mg acepromazine) anesthesia will be administered to all monkeys prior to all handling, bleeding, and vaccinations (IAW AC-11-13-01). For aerosol challenge the dose of Telazol will be 5 mg/kg.

5. Animals will be vaccinated (0.5 ml) with either vaccine or placebo intramuscularly in the left thigh using a 22-27 gauge needle attached to a 1 ml syringe, on days 0, and 21 (IAW AC-09-10-01).

6. The inoculation sites will be examined by either the PI or an investigator for any adverse reactions to the vaccines. Monkeys will be brought to front of cage and injection sites on the unanesthetized monkeys examined on days 1, 2, and 3 post-vaccination.

7. The animals will be bled immediately prior to vaccination, and then on days 7, 14, 21, 28, 35, 42, 50 and 58. The exact bleed amounts and proposed use is shown in Appendix 1. The methodology for dispatch of the various blood/serum samples collected is described in Appendix 4. In brief, this blood will be used to determine plasma antibody levels to both F1 and V and assess cell mediated immunity on each day. On day 58 (2 days prior to challenge) blood will be used for passive transfer, IgG levels to F1 and V, competitive ELISAs and cell cytoxocity assays, and cell mediated immunity. (IAW SOP # AC-13-00-01 and SOP # AC-13-30-01).

8. Following challenge, a maximum of 1.8 ml of blood will be drawn on days 3 – 7 for bacterial cultures, then 5 ml on days 7, 14, 21 and 28 days post-challenge for antibody levels. (IAW SOP # AC-13-00-01 and SOP # AC-13-30-01). Respiratory and heart rates will be determined (for the first 7 days post-challenge) at time of bleed and X-rays may be taken. Clinical signs will be documented (by either the PI or an investigator) twice per day for the first 7 days post-challenge, between 0800 and 0900 (immediately prior to anesthetization on bleed mornings) and between 1500 and 1600, and then once a day (mid morning) out to 28 days post-challenge (Appendix 2 & 3).

9. Any monkey that becomes a candidate for euthanasia will be bled (1.8–2 ml) for blood culture prior to death and a lung X-ray taken. (IAW SOP # AC-13-00 and SOP # AC-13-30-01)

10. Any monkey that shows clinical signs of disease in the time period 8-28 days post-challenge will be bled (1.8 ml) for bacterial culture. (IAW SOP # AC-13-00-01 and SOP # AC-13-30-01)

11. Baseline temperature and activity data will be collected a minimum of 10 days prior to challenge. Post-challenge temperature and activity data will be collected for a minimum of 28 days.

12. Initiation of procedures to remove survivors from the BSL-3 will occur when all survivors have shown no clinical signs of illness for approximately 6 weeks (in accordance with USAMRIID regulation 385-8).

The amount of blood to be drawn will be in accordance with the Veterinary Medicine Division Biomedical Investigator Laboratory Animal Handbook. (IAW SOP # AC-13-00-01 and SOP # AC-13-30-01)

B. Laboratory Animals Required and Justification:

1. Non-animal Alternatives Considered:

Alternatives were considered, but none are available. Efficacy trials of vaccines for human use require that immunized animals be challenged reproducibly with virulent organisms. Only in living, whole animals can the interaction of all functioning components of the immune system be examined. It is therefore necessary to perform vaccine efficacy studies in living experimental animals. There is currently no reliable predictor of immunity to plague (such as antibody titers) in vaccinated animals other than presenting them with a lethal challenge of *Y. pestis* and noting survival or death. As further studies on plague are carried out, an appropriate and specific parameter correlating with immunity may be found. Until that time, however, the only parameter to evaluate protective immunity is challenge with plague bacilli.

Vaccines designed to protect people from a biological warfare aerosol attack can be tested for safety in humans, but efficacy studies cannot be performed in humans for either ethical and/or practical reasons: aerosol exposure is not usually a natural route of exposure, the disease or intoxication is frequently deadly with no licensed available treatment, and the incidence of the naturally occurring disease is rare. Therefore, we have to rely on data from animal models for human disease.

The Food and Drug Administration (FDA) has amended its new drug and biological product regulations to allow appropriate studies in animals to provide substantial evidence of the effectiveness of new drug and biological products used to reduce or prevent the toxicity of chemical, biological, radiological or nuclear substances. This rule will apply when adequate and well-controlled clinical studies in humans cannot be ethically conducted and field efficacy studies are not feasible. In these situations, certain new drug and biological products that are intended to reduce or prevent serious or life-threatening conditions may be approved based on evidence of effectiveness derived from appropriate studies in animals and any additional supporting data. (Federal Register, Vol. 67, No. 105, May 31, 2002)

This is a pre-clinical study of a candidate vaccine; the purpose of this study is to gain information with regards to efficacy against an aerosol challenge and the potential for development of this candidate as a vaccine.

2. Animal Model and Species Justification:

M. fascicularis have been used in plague research since 1898 by the discoverer of the plague bacilli, Yersin [7]. Investigations over the last 100 years have shown that the disease in monkeys resembles that of humans. However, different species of monkeys vary in the susceptibility to *Y. pestis* and the relative resistance of *M. fascicularis* may be due to the various techniques employed to determine lethal doses of the plague bacilli and the various strains used. [8]. In addition, *M. fascicularis* have been used for testing vaccines and the immune response to vaccination has been studied extensively.

3. Laboratory Animals:

a. Genus & Species: *Macaca fascicularis* (cynomolgus macaque)

b. Strain/Stock: N/A

c. Source/Vendor: USAMRIID colony

d. Age: Adult

e. Weight: 4 - 8 kg

f. Sex: Male/female

g. Special Considerations: Monkeys should have a base-line lung X-ray, be negative for SRV, SIV and STLV and have no evidence of prior exposure to *Y. pestis* or detectable antibody to *Y. pestis*.

h. Other: N/A

4. Total Number of Animals Required: 22

a. Justification for the number of animals: With sample sizes of 10 per group experimental group and 2 per control group, only 100% response vs. 0% response can attain the statistical level of significance at the 95% confidence level (1-tailed Fisher exact test). However, if historical controls are used, a sample size of 10 will give sufficient power to determine score confidence limits of vaccine efficacy assuming a one-tailed alpha of .05. If 10 out of 10 animals survive and both controls die, this will establish vaccine efficacy of no less that 77% at the 95% confidence level. If 9 out of 10 survive, the corresponding efficacy will be no less than 65%, and if 8 survive, no less than 54% efficacy. Experimental conditions must be stable across experiments to allow pooling with this experiment. Historical controls (> 10) will be used in statistical analysis for comparison purposes. All controls receiving 50 - 200 LD50 have died to date. Two controls are being used in this experiment to ensure that the challenge dose is lethal.

5. Refinement, Reduction, Replacement:

a. Refinement: Respiratory function will be measured prior to aerosol challenge to refine the inhaled dose the animals will receive. The usage of radiotelemetry represents refinement in the use of research animals. Animals that have had telemetry devices implanted under previous approved protocols will be used whenever possible. The sera obtained in this study will be used to identify an in vitro correlate of immunity.

Reduction: Surviving monkeys will be returned to Veterinary Medicine Division for use in other studies at the end of this study. Extensive rodent studies have been undertaken to determine the optimal vaccine candidate, thus fewer monkeys are required to demonstrate efficacy of this vaccine.

c. Replacement: NA

C. Technical Methods:

1. Pain:

a. Pain category: (USDA Form 18-13)

(1) No Pain __0__

(2) Alleviated Pain or Distress__20__

(3) Unalleviated Pain or Distress __2__

The actual numbers of animals in the Unalleviated pain/No Pain Categories (if different from above) will be provided to the Chairman, LACUC, at the end of the study.

b. Pain Alleviation:

(1) Anesthesia: Telazol (2-9 mg/kg i.m.) or 10:1 ketamine/acepromazine mixture @ 0.1ml/kg (9mg/kg ketamine and 0.1 mg acepromazine) anesthesia will be administered to all monkeys prior to all handling, surgery, bleeding, immunizations, aerosol challenge and euthanasia. All Telazol injections will be given in the caudal thigh muscle (with a 23-27 ga. 5/8"-1") needle attached to a 1.0 ml syringe) by trained technicians and investigators. Critically ill monkeys will be euthanized by deep anesthesia with 6-9 mg/kg Telazol (IM) followed by an intravenous or intracardiac overdose of sodium pentabarbitol.

(2) Analgesia: The monkeys will receive Buprenorphine (0.3 mg/ml) administered IM using a 1 ml syringe with a 23-27 gauge, ½-1 inch needle in the caudal thigh muscles to provide postoperative analgesia in accordance with SOP # AC-09-10-10. It will be administered at a dosage of 0.01 mg/kg BID for 48 hours post-op and as necessary based on clinical signs as determined by the veterinarian.

(3) Paralytics: N/A

c. Alternatives to Painful Procedures:

(1) Sources Searched: Medline, AGRICOLA, BIOSIS

(2) Date of Search: 1966 – 2003; 29 May 2003

(3) Key Words of Search: pneumonic plague, vaccine, aerosol, F1 antigen, V antigen, alternative, welfare, method, model, in vitro, pain, distress, simulate, replacement, refinement, reduction

(4) Results of Search: The principal investigator did not find any alternatives to the painful procedures involved in doing plague vaccine efficacy studies during this search.

Painful Procedure Justification: All 22 animals will receive anesthesia prior to being bled; 2 animals (controls) are expected to succumb to the disease and die or be euthanized and are in the unalleviated pain and distress category. All infected nonhuman primates that show overt clinical signs of plague will presumably be in distress as a result of the infection. The use of anesthetics or analgesics during the course of infection to alleviate symptoms might introduce complicating

variables that could compromise the results of the study. Analgesics may interfere with evaluation of efficacy as important subtle clinical signs could be masked. However, infected monkeys that have severe difficulty breathing with fluid on the lung, will be euthanized. The attending veterinarian was consulted regarding painful procedures.

Opioids are reported to be associated with histamine release; steroids may reduce the inflammatory response; and other analgesics may interfere with cytokine release. Narcotic analgesics can cause histamine release and respiratory depression and bradycardia and nonsteroidal anti-inflammatory drugs directly interfere with the inflammatory process that is a critical component in the pathogenesis of *Y. pests* infection.

Opioid analgesics are well documented to cause substantial respiratory depression in nonhuman primates, to include Butorphanol, an opioid commonly used for pain alleviation in laboratory animals [9, 10]}. Opioid drugs administered at typical analgesic dosages have important effects on intestinal motility as they cause increased frequency of nonpropulsive phasic contractions and a significantly decreased frequency of propulsive migrating contractions in nonhuman primates [11, 12]. A recent study in humans has also shown that opioids have a significant effect on thermoregulatory control and that concentrations of opioids commonly observed in critical care patients significantly inhibit the manifestation of fever [13].

2. Prolonged Restraint: N/A

3. Surgery:

a. Procedure: Transducers will be placed subcutaneously on the dorsum of the animal between the scapula, just off the midline at the level of the inferior edge of the scapular blade. Surgery will be conducted under strict sterile procedures and surgical plane of anesthesia will be maintained with isoflurane and oxygen. Once the incision is made, the skin will be undermined to form a pocket in the subcutis for the telemetry device. Following placement of the telemetry device the incision will be closed using absorbable suture of an appropriate size in a continuous pattern (IAW SOP 10-03-00).

b. Pre- and Postoperative Provisions: Pre-operative provisions: Monkeys will be held off feed overnight prior to surgery. Surgical preparation consists of premedication with buprenorphine (0.01-0.03mg/kg, IM), induction of anesthesia with Telazol (5 mg/kg; IM) or 10:1 ketamine/acepromazine mixture @ 0.1ml/kg (9mg/kg ketamine and 0.1 mg acepromazine), endotracheal tubation, hair removal of the dorsum between the scapulae followed by a standard surgical scrub. Animals will be kept warm using thermoblankets (recycling water) during surgical procedures.

Post-operative provisions: After closure of last skin incision, monkeys will be extubated after swallow reflex has returned, placed back into his/her cage and monitored until sitting upright is accomplished at which time the monkeys will be given post-surgical analgesia. The surgical sites will be observed at least once daily for any signs of complication, including evidence of excess inflammation, infection, and dehiscence at the incision site.

c. Location: Surgical procedures will be performed in the NHP treatment room of Bldg. 1412. However, animals will initially be induced with anesthesia and recover from surgery

(under technician or surgeon observation) in the animal's own cage. All procedures described as surgical preparation will occur in the surgical prep area immediately adjacent to the treatment room. Surgery starts with the skin incision and ends with skin closure.

d. Surgeon/Qualification: MAJ Pedro Rico, MAJ Mallory Tate and CPT Christopher S. Gamble, all of the Veterinary Medicine Division, will perform the surgeries. Their qualifications are listed in section V.H. "Investigator & Technician Qualifications/Training."

e. Multiple Survival Surgery Procedures: It is not anticipated that the limited supply and battery life of the implants will necessitate the removal of implants from surviving animals. However, should this become an issue, it will only be dealt with on an animal-by-animal basis through an addendum to this protocol.

4. Animal Manipulations:

a. Injections: Anesthesia (Telazol 2-5 mg/kg) or 10:1 ketamine/acepromazine Mixture @ 0.1ml/kg (9mg/kg ketamine and 0.1 mg acepromazine) injections will be administered i.m. into the caudal thigh muscle of nonhuman primates, with a 23-27 ga. 5/8"- 1" needle attached to a 1 ml syringe. The plague vaccine and placebo (0.5 ml) will be given i.m. using a 23-27 ga. 5/8"- 1" needle attached to a 1 ml syringe, in the left caudal thigh muscle.

b. Biosamples: Aseptic techniques and preparations will be used throughout these procedures. Blood specimens will be collected from all anesthetized monkeys with a 21-23 ga. 1.5" needle via a peripheral vein. (IAW SOP # AC-13-00-01 and SOP # AC-13-30-01)

Blood specimens will be taken throughout the study period IAW the table in Appendix 1.

c. Animal Identification: All monkeys will have a microchip implanted and have a legible tattoo.

d. Behavioral Studies: N/A

e. Other procedures: Plethysmography (SOP # TX-02-13-01) and aerosol challenge (SOP # TX-02-06-00, TX-02-08-00, TX-02-15-00, and TX-02-16-00) will be conducted IAW the referenced SOPs for NHPs All monkeys will be radiographed (dorso-ventral and lateral views of the lungs) prior to being placed on study and monkeys that are candidates for euthanasia will be radiographed.

f. Shared tissues: Blood and serum from vaccinated/challenged surviving monkeys and tissues from the controls and any vaccinated monkeys that may die will be shared with other USAMRIID investigators.

5. Adjuvants:

Aluminum hydroxide (Alhydrogel) is the only adjuvant in licensed vaccines FDA-

approved for human use. The site of injection will be observed for 3 consecutive days for any adverse reaction.

6. Study Endpoint:

The following clinical signs will be monitored: weakness, progressive state of depression, inappropriate responses to external stimuli, and forced abdominal respiration with rales, temperature and pulse O_2 levels monitored. Euthanasia will be performed to prevent unnecessary pain or distress when clinical signs have been confirmed (Cumulative Score of ≤ 5, Appendix 2 or when abdominal respiration with rales is present and fluid found radiographically in the lungs).

All vaccinated animals that survive 42 days post lethal challenge will be considered protected against plague. They will be "grayed out" of the BSL-3 and returned to the Veterinary Medicine Division at the end of the study.

Complete gross and histopathologic postmortem examinations will be performed in accordance with USAMRIID SOP # PT-02-15-00 on all monkeys that die or are euthanized .

7. Euthanasia:

Monkeys that become candidates for euthanasia will be anesthetized with Telazol anesthesia (6-9 mg/kg IM) or 10:1 ketamine/acepromazine mixture @ 0.1ml/kg (9 mg/kg ketamine and 0.1 mg acepromazine). After a complete loss of sensation, monkey will be administered an overdose of Euthanasia solution (Euthasol®), intravenously or intracardiacally (using a 23 gauge needle) at a dose of 200 mg/kg. (IAW AC-11-03-01) Euthanasia will be confirmed by the absence of a heartbeat after 5 minutes. Blood will be collected for culture (1.8 ml). Decision to euthanize will be made by either by Dr. Pitt, MAJ Anderson, or the attending veterinarian and performed by them or a trained animal care specialist.

D. Veterinary Care:

1. Husbandry Considerations: Cynomolgus macaques will be identified by chest tattoo and microchip, housed individually in stainless steel cages with squeeze capability, and fed a commercial monkey ration supplemented with fruit daily. Water will be provided ad libitum via automatic waterers. Post-challenge, high water content fruits will be provided and a water bottle will be placed low in the cage to allow for easy access. Commercially available electrolyte replacement drinks and floor-placed water bowls will be provided as necessary.

a. Study Room: Rm 120, Bldg. 1412

b. Special Husbandry Provisions: Personnel handling nonhuman primates during exposure and for the subsequent 42 days will wear the appropriate respiratory protection (PAPR) and Tyvek suits, double gloves, and booties.

2. Attending Veterinary Care: The Veterinary Medicine Division animal caretaker will examine the animals daily for general health, husbandry conditions and humane treatment. Following vaccination, the injection sites will be checked on days 1, 2 and 3 for any adverse reactions to the

adjuvant.
Following challenge, the animals will be observed twice daily for clinical signs by the principal investigator and qualified Veterinary Medicine Division personnel. Animals that are critically ill will be euthanized promptly. Decision to euthanize will be made by the principal investigator or attending veterinarian.

3. Enrichment Strategy:

a. Dogs: N/A

b. Nonhuman Primates: In accordance with Veterinary Medicine Division's SOP AC-06-36-00.

E. Explanation for Exception to the AWA, Guide and /or Established Policies:

Portions of this protocol will be performed under BSL-3 conditions, therefore requiring an exception to standard nonhuman primate cage sanitizing procedures, due to the logistical confinements and safety considerations of moving cages in and out of the containment suites. Instead of cages and racks being sanitized a minimum of every two weeks in a mechanical washer, they will be manually sanitized in place during the containment portion of the study. Daily cleaning and husbandry will be performed according to standard procedures. Procedures will be performed in a manner to ensure that animals remain dry at all times and do not come into contact with any sanitizing agents used. Cages will be continuously evaluated by the caretaker and when, in the opinion of the caretaker and the Chief of the Department of Animal Husbandry, cages can no longer be appropriately sanitized in place, they will be replaced with mechanically sanitized caging (IAW SOP # AC-02-08-01).

There is no vaccine available for plague, thus, once the animals are exposed to Y. pestis they will be isolated from the rest of the BSL-3 suite. Euthanasia, if necessary, will therefore be performed in the animal room. Prior to any euthanasia procedures, every precaution will be taken to ensure stress to the other occupants of the room will be minimized. Actions taken will include anesthetizing the candidate for euthanasia, working in a corner of the room to minimize the view, and turning cages of other occupants.

F. Data Analysis: Data will be entered into an electronic spreadsheet (Excel). Data sets will be compatible with computerized statistical programs. The data entry format will be standardized to minimize entry errors, and quality control will be incorporated into data entry. Data will be analyzed by descriptive statistics (means, standard deviations and confidence intervals) on a computerized statistical program (BDMP, SAS, or SPSS). Representative graphs and charts will be used to illustrate these statistics. Efficacy of the vaccine will be determined by the lower 95% confidence level on the survival rate for each challenge strain using the method of "score confidence intervals" which have been shown recently to have coverage probabilities closer to the true level than "exact" methods. Serum antibody levels will be analyzed for mean time trends.

Telemetry temperature data will be adjusted for diurnal variation by time series models and

analyzed for mean time trends. Confidence intervals will be at the 95% level.

G. Record Keeping: Records will be kept in standard USAMRIID laboratory notebooks and 3-ring binders. Daily records will be kept on survival and clinical signs collected on the animals post-challenge. All this information should be logged in the individual animal medical records as it relates to relevant information on the health of the animal. Procedures for preparation of vaccine dilutions, vaccination logs, bleed logs, and observation/ morbidity/mortality logs will be stored with the study records.
Experimental protocols, summary of data, statistical analysis, tabular calculations, graphic analysis of the data, and conclusion of the experiment will be saved with the study records.

H. Investigator & Technician Qualifications/Training:

Dr. Louise Pitt, Ph.D., has 25 years experience in biomedical research with most common laboratory animals including the rabbit, guinea pig and nonhuman primate. This experience includes exposing animals to aerosols in various types of equipment. She has had 14 years of experience at USAMRIID, working with the aerosol facility and is familiar with the head and nose-only exposure systems used in this Institute. She has published and presented her research nationally and internationally. Dr. Pitt will be responsible for overseeing the study and the telemetry, for supervising the clinical monitoring of the monkeys, making decisions on euthanasia and performing euthanasia.

MAJ Mallory K. Tate, DVM, has five years experience in clinical animal medicine. He has 2 years of experience using nonhuman primates in animal manipulations, surgery, drug administration, clinical evaluations, and euthanasia. He will assist with the surgery, animal manipulations and may perform euthanasia, and clinical observations

MAJ Pedro J. Rico, DVM, MPH has 20 years experience in laboratory animal medicine field with 8 years experience working with different species of nonhuman primates. Currently he is Chief, Department of Laboratory Animal Medicine. He completed the LAM Residency at USUHS in Jun 2000 and achieved ACLAM Diplomate status in July 2002. He is experienced in clinical evaluation and treatments, surgery, blood collection, anesthesia and euthanasia procedures. Has experience working in BSL 3 and BSL 4 containment suites. CPT Christopher S. Gamble has a DVM degree with 6 years experience in clinical medicine and surgery, and 7 years experience working in the laboratory animal field, primarily in an academic setting as a graduate student and animal caretaker. Dr. Gamble will act as surgeon or anesthesiologist for this experiment. Dr. Gamble will also assist in performing animal manipulations (handling, inoculating, bleeding, anesthetizing, euthanasia, and necropsy).

MAJ Anderson, DVM, Ph.D, ACVPM has 14 years experience in veterinary medicine. His experience encompasses housing, management, manipulation, restraint, medical diagnostics and medical treatment of NHPs. He has received training on handling of nonhuman primates by the Veterinary Medical Division at USAMRIID. He will oversee all aspects of this protocol, to include monitoring the animals.

Mr. Keith Esham is certified by American Association of Laboratory Animal Science at the Laboratory Animal Technologist (LATG) level. He has 6 years of experience at USAMRIID handling nonhuman primates, rabbits, guinea pigs, hamsters, rats and mice. This includes

administering anesthesia, phlebotomy, emergency procedures, animal observations and euthanasia at BSL-3 and BSL-4 levels of containment. He will assist with all animal manipulations and will also perform euthanasia.

David Dyer is a technician with the Department of Aerobiology and Product Evaluation, Division of Toxinology and Aerobiology. Mr. Dyer has experience with inhalation toxicology research, including experience with various exposure systems. He has been trained in handling of NHPs including restraint, administration of anesthesia, bleeding via femoral triangle, and observing for signs of clinical illness. It is expected that throughout the study period he will handle live animals and will assist in injections, aerosol challenges, biosample collection, and animal observation. He will be responsible for the monitoring the telemetry data collection, will assist with all animal manipulations to include treatments, aerosol exposure, handling of the animals, and monitoring for clinical signs of infection.

Trained animal care specialists assigned to the Animal Medical Section of the Veterinary Medicine Division will assist the investigators with the monitoring and handling of the animals. These animal care technicians have been trained in all aspects relating to the manipulation and observation/health monitoring of nonhuman primates and will perform all the bleedings.

Formal euthanasia training has been provided and is documented in the Veterinary Medicine Division's training files.

Either Dr. Pitt, MAJ Anderson, the attending veterinarian or the trained animal care specialists will euthanize the animals.

The other investigator will not handle the animals.

I. Time Lines:

1. Estimated Start Date: August 2003

2. Estimated Completion Date: December 2004

VI. BIOHAZARD\SAFETY:

Containment level: BSL-3

Agents used: *Yersinia pestis*, strain CO92

Registration Number: 2224

VII. EXTRAMURAL COLLABORATION: DSTL, MOD, Porton Down, Salisbury, UK

IX. ASSURANCES: As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a deviation is specifically approved by the LACUC.

B. Duplication of Effort: I have made a reasonable good faith effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified statistician to evaluate the statistical design or strategy of this proposal, and that the "minimum number of animals needed for scientific validity are used."

D. Biohazard\Safety: I have taken into consideration and I have made the proper coordinations regarding all applicable rules and regulations concerning radiation protection, biosafety, recombinant issues, etc., in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures\manipulations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures\manipulations.

F. Responsibility: I acknowledge the inherent moral and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" which the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible, and conducting humane and lawful research.

M. Louise M. Pitt

(Principal Investigator Signature)

G. Painful Procedures: A signature for this assurance is required by the P.I. only if the research being conducted will cause more than slight or momentary pain or distress (see page 2.V.C.1.a.(2) & (3), Column D or E by USDA classification:

I am conducting biomedical experiments that may potentially cause more than momentary or slight pain or distress to animals that WILL NOT be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures, however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

M. Louise M. Pitt

(Principal Investigator Signature)

H. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review, and is consistent with good scientific research practice.

Chris Bythrow

(Division Chief Signature)

MAJ MILLARD 3-4261

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X. Enclosures:

A. Bleed/manipulation Schedule (Appendix 1)

B. Clinical Signs Score Sheet (Appendix 2)

C. Pain Assessment Guidelines (Appendix 3)

D. Logistics (Appendix 4)

Appendix 1 - CALENDAR OF SIGNIFICANT EVENTS

DAY	EVENT
0	Bleed (10 ml)/Vaccination
7	Bleed (10 ml)
14	Bleed (10 ml)
21	Bleed (10 ml) /Vaccination
28	Bleed (10 ml)
35	Bleed 10 ml)
42	Bleed (10 ml)
50	Bleed (10 ml)
58	Bleed (20 ml)
60	Challenge
63	Bleed (1.8 ml)
64	Bleed (1.8 ml)
65	Bleed (1.8 ml)
66	Bleed (1.8 ml)
67	Bleed (6.8 ml)
74	Bleed (5 ml)
81	Bleed (5 ml)
88	Bleed (5 ml)

NOTE: ALL SCHEDULED EVENTS MUST OCCUR WITHIN ± 2 DAYS OF THIS SCHEDULE

Appendix 2: Post-Exposure Check List for Clinical Signs

Protocol #:_____ Date:_____AM / PM

Day Post-Exposure:_____ Monkey Tattoo Number:_____

Weight:_____ kg _____ Pulse O_2:_____ % _____
initials initials

RR: _____ rpm _____ HR:_____ bpm _____
initials initials

Food Consumption

Biscuits		Fruit	
Given (am)	Eaten (pm)	Given (am)	Eaten (pm)

Subjective Assessment

Activity 1 - 5	Behavior 1 - 5	Stimuli response 1 - 5	Respiratory Distress 1 - 5	Cumulative Score

Key: Activity: Normal – 5; active – 4; slow active – 3; sluggish –2; inactive – 1
Behavior: Normal – 5; antisocial – 4; depressed – 3; hunched, back to observer – 2; ignoring all – 1
Stimuli response: Normal – 5; enter room – 4; approach cage – 3; rattle cage – 2; pinch –1
Breathing: Normal – 5; rapid – 4; abdominal breathing – 3; dyspnea – 2; rales –1

Euthanasia criteria: Cumulative score of 5 OR evidence of abdominal breathing with rales / fluid on the lung

Comments:

Subjective Assessment performed by:_____ (signature)

Appendix 3 - PAIN ASSESSMENT GUIDELINES

COMMON CLINICAL SIGNS INDICATING PAIN, DISTRESS, OR DISCOMFORT IN EXPERIMENTAL ANIMALS

SYSTEM	SIGNS
Cardiovascular	Heart rate altered: pulse quality affected: Peripheral circulation decreased, blue and cold extremities (ears, paws).
Respiratory	Abnormal breathing pattern, rate and depth altered, labored, panting, nasal discharge.
Digestive	Bodyweight loss or poor growth: feces altered in volume,color or consistency (e.g.,black with blue: pale, lack of bile pigments, undigested food: diarrhea/constipation): vomiting, jaundice, salivation.
Nervous	Twitching, fitting, tremors, convulsions, musculoskeletal paralysis, pupils dilated, shivering, (locomotory) hyperaesthesia, reflexes sluggish or absent: unsteady gait, lameness, muscle flaccidity, rigidity or weakness, protecting affected area such as "boarding" abdomen or reluctant to move a limb (e.g., arthritis).
Miscellaneous	Any abnormal swelling protrusion (hernia, rupture) or abnormal discharges from natural orifices; raised body temperature. Dehydration; sunken eyes, skin tents. Urine; specific gravity increase, decrease in volume.

From: Morton, D.B. and P.H.M. Griffiths (1985) Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment. Vet. Rec. 116: 431-436.

Challenge Date: 28-Oct-03
PI: Pitt
AED: 2003-403
Agent: Y. Pestis

Run #	Animal ID	Start Conc [cfu/ml]	Min Vol [ml]	Exp Time [s]	Wash Time [s]	AGI Vol [ml]	AGI Q [ml/min]	AGI Conc [cfu/ml]	Aero Conc [cfu/ml]	Dose [cfu]	Dose [LD50]	Spray Factor
1	C440	1.8E+06	755	872	300	10	6000	9.7E+03	0.82	1.2E+04	35	6.3E-07
2	78-143	1.8E+06	442	1475	300	10	6000	5.9E+04	3.32	4.3E+04	127	2.3E-06
3	49-470	1.8E+06	834	791	300	10	6000	3.1E+04	2.84	4.3E+04	126	2.2E-06
4	C512	1.8E+06	1036	641	300	10	6000	2.1E+04	2.23	3.6E+04	106	1.9E-06
5	41-318	1.8E+06	1709	397	300	10	6000	1.9E+04	2.77	5.5E+04	161	2.9E-06
6	94985	1.8E+06	528	1238	300	10	6000	5.5E+04	3.60	4.9E+04	142	2.5E-06
7	41-384	1.8E+06	950	698	300	10	6000	3.6E+04	3.57	5.6E+04	165	2.9E-06
8	59-213	1.8E+06	897	737	300	10	6000	3.7E+04	3.57	5.5E+04	161	2.9E-06
9	49-516	1.8E+06	647	1014	300	10	6000	3.6E+04	2.71	3.8E+04	112	2.0E-06
10	30-318	1.8E+06	504	1296	300	10	6000	5.3E+04	3.32	4.5E+04	130	2.3E-06
11	41-363	1.8E+06	610	1074	300	10	6000	4.3E+04	3.13	4.4E+04	127	2.3E-06
Avg									Avg:	**4.3E+04**	**126**	**2.2E-06**
									Stdev:	**1.2E+04**	**36**	

Challenge Date: 29-Oct-03
PI: Pitt
AED: 2003-404
Agent: Y. Pestis

Run #	Animal ID	Start Conc [cfu/ml]	Min Vol [ml]	Exp Time [s]	Wash Time [s]	AGI Vol [ml]	AGI Q [ml/min]	AGI Conc [cfu/ml]	Aero Conc [cfu/ml]	Dose [cfu]	Dose [LD50]	Spray Factor
1	79-27	4.6E+06	467	709	300	10	6000	6.2E+04	6.18	4.9E+04	141	2.0E-06
2	39-38	4.6E+06	588	567	300	10	6000	4.4E+04	5.07	4.3E+04	126	1.8E-06
3	C694	4.6E+06	398	828	300	10	6000	7.0E+04	6.18	4.6E+04	135	1.9E-06
4	49-458	4.6E+06	604	553	300	10	6000	4.4E+04	5.12	4.4E+04	128	1.8E-06
5	99-310	4.6E+06	914	372	300	10	6000	2.9E+04	4.32	4.4E+04	129	1.8E-06
6	59-25	4.6E+06	552	603	300	10	6000	4.4E+04	4.91	4.1E+04	119	1.7E-06
7	C616	4.6E+06	679	494	300	10	6000	3.4E+04	4.28	3.8E+04	112	1.6E-06
8	107-720	4.6E+06	523	635	300	10	6000	4.1E+04	4.42	3.6E+04	105	1.5E-06
9	C470	4.6E+06	500	664	300	10	6000	4.4E+04	4.60	3.7E+04	108	1.5E-06
10	49-544	4.6E+06	838	405	300	10	6000	3.8E+04	5.34	5.3E+04	153	2.1E-06
		Mean:	**606**						Mean:	**4.3E+04**	**126**	**1.8E-06**
		Stdev:	**163**						Stdev:	**5.2E+03**	**15**	**2.1E-07**
		C of V:	**0.27**						C of V:		**0.12**	

USAMRIID PATHOLOGY REPORT

Accession Number	Animal Number	Protocol Number	Investigator	Division
031476	41-384	F03-11	PITT	TOXINOLOGY

Sacrifice Method	Species	Strain	Sex	Project #
	MONKEY	CYNO	MALE	02-4-AA-003

Weight	Date of Challenge	Date of Death	Date Necropsied	Report Date	Prosector
5.9KG	~10/27/03	11/3/03	11/3/03	3/5/2004	MAJ BATEY

KeyWords
plague, UK vaccine

History

This adult male cynomolgus monkey received vaccination (40 μg F1 + 40 μg V in 0.5 ml 20% v/v Alhydrogel) then was challenged with approximately 100 LD50 of the F1 positive *Y. pestis* strain, CO92. The challenge dates for this group were 27 or 28 October, 2003. This monkey was presented for necropsy on the day of death. A complete necropsy was performed but only select tissues were taken for histopathologic examination, according to instructions from the PI.

Pathological Summary

Gross Pathology:
The trachea and bronchi are filled with moderate amounts of pink "bloody" froth. The lungs are diffusely wet and heavy with edema. Diffusely affecting all lobes of lung are multifocal to coalescing areas of dark purple discoloration. There are multifocal fibrous adhesions between the left lung lobes and the chest wall. There is a small amount of reddish, clear edema fluid in the thoracic cavity. There is a small ~3-4 mm focus on the surface of the right adrenal gland.

Gross Diagnosis: Severe diffuse bronchopneumonia

The following organs were weighed at necropsy:

Organ weights (grams):
Thyroid gland: 0.8
Thymus: atrophic, not weighed
Lung (with bronchi): 165.6
Spleen: 11.1
Liver: 165.8
Adrenal glands L: 1.1 R: 0.9
Kidneys L: 12.6 R: 12.7
Brain: 62.1

Microscopic Diagnoses:
1. Thyroid gland: Follicular cysts, multiple.
2. Trachea: Tracheitis, chronic, diffuse, mild.
3. Trachea: Tracheitis, subacute, multifocal, minimal, with epithelial degeneration and necrosis.
4. Lung, diaphragmatic and apical lobes: Pneumonia, subacute, necrotizing and suppurative, diffuse, severe, with bronchiolar epithelial necrosis and loss, multifocal hemorrhage, diffuse vascular congestion and edema, multifocal vasculitis, multifocal fibrinous pleuritis, and myriad intra-alveolar bacilli, etiology consistent with *Yersinia pestis*.
5. Adrenal gland, cortex: Hyperplasia, nodular, multifocal, mild.
6. Bronchus: Bronchitis, subacute diffuse, minimal.
7. Mandibular lymph node: Sinus histiocytosis, diffuse, moderate, with erythrophagocytosis.
8. Mesenteric lymph node: Sinus histiocytosis, diffuse, marked, with erythrophagocytosis.
9. Tongue; parathyroid gland; gallbladder; liver; spleen; kidneys; thymus; brain: No significant lesions.

Pathological Summary Continued...

COMMENTS: The lesions observed in this animal are consistent with *Yersinia pestis* infection. The primary cause of death is severe bacterial pneumonia. The bronchus was only minimally inflamed but was filled with septic exudate from the lung. The adrenal lesion noted at necropsy was cortical nodular hyplasia, an incidental finding. The tracheobronchial lymph node was not found in the trimmed sections. The urinary bladder was also missing at trim. Histologic evidence of septicemia was not found in this case. The time to death and lesions in this case were not that different from the two unvaccinated controls.

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USAMRIID PATHOLOGY REPORT

Accession Number	Animal Number	Protocol Number	Investigator	Division
031474	99-310	F03-11	PITT	TOXINOLOGY

Sacrifice Method	Species	Strain	Sex	Project #
	MONKEY	CYNO	FEMALE	02-4-AA-003

Weight	Date of Challenge	Date of Death	Date Necropsied	Report Date	Prosector
3.3KG	~10/27/03	11/2/03	11/2/03	3/1/2004	MAJ BATEY

KeyWords
plague, UK vaccine

History

This adult female cynomolgus monkey received a placebo vaccination (saline/alhydrogel) then was challenged with approximately 100 LD50 of the F1 positive *Y. pestis* strain, CO92. The challenge dates for this group were 27 or 28 October, 2003. This monkey was presented for necropsy on the day of death. A complete necropsy was performed but only select tissues were taken for histopathologic examination, according to instructions from the PI.

Pathological Summary

Gross Pathology:
There is a moderate amount of pink froth within the trachea and bronchi. The lungs are diffusely wet and heavy with multifocal to coalescing dark red to purple foci of variable sizes; all lung lobes are involved. The lungs failed to collapse upon opening the chest cavity. There is a small 2 x 4 cm cutaneous bruise along the L inner thigh (venipuncture site?). No other lesions were noted.

Gross Diagnosis: Severe diffuse bronchopneumonia

The following organs were weighed at necropsy:

Organ weights (grams):
Thyroid gland: 0.5
Thymus: 2.0
Lung (with bronchi): 67.7
Spleen: 4.5
Liver: 105.2
Adrenal glands L: 0.7 R: 0.5
Kidneys L: 9.0 R: 9.2
Uterus with cervix: 13.0
Brain: 72.4

Microscopic Diagnoses:
1. Thyroid gland: Intravascular bacilli, multifocal, with focal fibrin thrombus.
2. Trachea: Tracheitis, chronic, diffuse, moderate to marked.
3. Trachea: Tracheitis, subacute, multifocal, mild, with mucosal erosion and bacilli.
4. Lung, diaphragmatic lobe: Pneumonia, diffuse, moderate to marked, with alveolar flooding, myriad intra-alveolar bacilli, diffuse mild subacute inflammation, mild multifocal bronchiolar epithelial erosion, minimal multifocal necrosis, minimal multifocal hemorrhage, and mild multifocal pleuritis.
5. Lung, apical lobe: Pneumonia, subacute, suppurative, multifocal, moderate to marked, with mild multifocal necrosis, mild multifocal hemorrhage, alveolar flooding, myriad bacilli, and mild multifocal pleuritis.
6. Tracheobronchial lymph node: Lymphadenitis, subacute, suppurative, multifocal to coalescing, moderate, with necrosis and myriad bacilli.

Pathological Summary Continued...

7. Bronchus: Bronchitis, subacute to chronic, diffuse, mild with multifocal erosion and bacilli.
8. Liver: Hepatitis, acute, multifocal, random, minimal to mild, with necrosis, bacilli, and intra-sinusoidal fibrin/bacterial thrombi.
9. Spleen, red pulp: Splenitis, subacute, diffuse, mild, with occasional bacilli.
10. Adrenal glands: Adrenalitis, acute, multifocal, random, minimal to mild with necrosis.
11. Mandibular lymph node: Draining subacute inflammation and minimal hemorrhage.
12. Thymus, blood vessels: Intravascular bacilli, multifocal, few.
13. Urinary bladder; gallbladder; kidneys; uterus; cervix, mesenteric lymph node; vagina; brain: No significant lesions.

COMMENTS: The lesions observed in this animal are consistent with *Yersinia pestis* infection. The primary cause of death was asphyxiation due to compromise of the lower respiratory system. The lung lesions varied somewhat from lobe to lobe and most were included in the microscopic diagnosis. Diffuse marked congestion and perivascular/interstitial edema was also observed in all lung sections. One blood vessel within the diaphragmatic lobe contained the beginnings of a neutrophilic and fibrin thrombus. In the apical lobes, there is bacterial colonization (large colonies) of perivascular and interstitial connective tissue and the pleura that was not observed in the diaphragmatic lobe sections. Alveolar flooding is used to describe a primarily protein rich edema fluid admixed with few erythrocytes or leukocytes. In the lung, bacteria are primarily extracellular but can be found multifocally within macrophages; small numbers of bacilli are observed within the blood vessels (septicemia). In the liver, bacilli are observed free within sinusoids, in Kupffer cells, in fibrin thrombi and occasionally within the affected parenchyma. The splenic red pulp is diffusely expanded by congestion and edema and contains a mild increase in leukocytes; bacteria can occasionally be observed within histiocytes and rarely, free within the blood. Septicemia, with bacterial colonization of many different organs is common following a respiratory infection with *Yersinia pestis*. The tongue was missing at trim.

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USAMRIID PATHOLOGY REPORT

Accession Number	Animal Number	Protocol Number	Investigator	Division
031378	49	F03-11	PITT	VET MED

Sacrifice Method	Species	Strain	Sex	Project #
FOUND DEAD	MONKEY	CYNO	MALE	02-4-AA-003

Weight	Date of Challenge	Date of Death	Date Necropsied	Report Date	Prosector
4.0 KG	UNKNOWN/NA	10/22/03	10/22/03	12/11/2003	BATEY

KeyWords
plague

History

Male, 19 y.o.a. Cynomolgus macaque found dead in cage at 0800 rounds on 10-22-03. Presented 10-21-03 QAR, responsive to food treats but was uncomfortable moving. Animal Previous medical history: has been on one previous protocol with exposure to VEE (F01-10). No other exposures noted. Vaccinated with plague vaccine for current protocol on 9-19-03. Telemetry device implanted 7-18-01. Has been negative for TB, SRV, SIV, and STLV-1 since entering colony. Animal was slow to come up from anesthesia (Telezol) following protocol bleed on 10-17-03.

Pathological Summary

Necropsy Findings:
Presented with an adult male Cynomolgus macaque with moderate to severe generalized subcutaneous edema especially of the face and thorax. The pectoral musculature is moderately atrophic and pale. There is a large amount of clear, yellow fluid within the pleural cavity. The lungs are atelectic and floating in the fluid. The liver is diffusely pale tan, with a mildly cobblestoned surface and a granular texture on cut sections. There is a small amount of clear, yellow abdominal cavity fluid. There is a moderate degree of periodontal disease and dental attrition.

Microscopic Diagnoses:
1. Heart: Cardiomyopathy, multifocal, moderate, with degeneration, atrophy, fibrosis, and minimal multifocal chronic inflammation.
2. Lung: Septal fibrosis and thickening, diffuse, mild, with intra-alveolar hemosiderophages ("heart failure" cells).
3. Liver: Fibrosis, centrilobular, multifocal, moderate, with centrilobular hepatocellular vacuolar degeneration, atrophy and loss, and multifocal necrosis.
4. Liver: Congestion and edema, diffuse, moderate.
5. Skin: Subcutaneous edema, diffuse, moderate.
6. Mandibular lymph node: Sinus histiocytosis, multifocal, mild with erythrophagocytosis and draining hemorrhage.
7. Mediastinal lymph node: Sinus histiocytosis, diffuse, moderate, with erythrophagocytosis.
8. Nares: Rhinitis, chronic, multifocal, moderate with erosion and ulceration.
9. Trachea: Edema, diffuse, mild with lymphatic dilation.
10. Tracheal cartilage: Mineralization, diffuse, moderate.
11. Thyroid, follicles: Cystic change, multifocal, moderate.
12. Parathyroid: Cyst, focal.
13. Mesenteric lymph node: Sinus histiocytosis, diffuse, marked, with hemosiderosis.
14. Axillary lymph node: Draining hemorrhage and edema, mild with erythrophagocytosis.
15. Pancreatic lymph node: Draining edema, diffuse, marked.
16. Bone marrow: Myeloid atrophy, diffuse, moderate.
17. Skeletal muscle, quadriceps: Sarcocyst, focal.
18. Skeletal muscle, quadriceps: Fibrosis and loss, focal, moderate, with minimal chronic inflammation.
19. Tongue; tonsil; parotid salivary gland; lip; larynx; esophagus; spleen; gallbladder; kidneys; urinary bladder; testes; prostate; urethra; adrenal glands; stomach; duodenum; pancreas; jejunum; ileum; cecum; sciatic nerve; brachial plexus; brain; eyes: No significant lesions.

Comments:
The primary cause of death was determined to be acute cardiac decompensation secondary to chronic heart disease. The lung lesion is the result of chronic congestive heart failure. The liver had both a chronic (centrilobular fibrosis) and a more acute (centrilobular vacuolar degeneration) lesion caused by heart failure. Herpes B was considered as a cause of the ulcerative rhinitis but no viral inclusion bodies were found. Many of the other lesions coded above are common findings in the aging macaque.

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USAMRIID PATHOLOGY REPORT

Accession Number	Animal Number	Protocol Number	Investigator	Division
031475	49-470	F03-11	PITT	TOXINOLOGY

Sacrifice Method	Species	Strain	Sex	Project #
	MONKEY	CYNO	MALE	02-4-AA-003

Weight	Date of Challenge	Date of Death	Date Necropsied	Report Date	Prosector
7.78KG	~10/27/03	11/1/03	11/1/03	3/5/2004	MAJ BATEY

KeyWords
plague, UK vaccine

History

This adult male cynomolgus monkey received a placebo vaccination (saline/alhydrogel) then was challenged with approximately 100 LD50 of the F1 positive *Y. pestis* strain, CO92. The challenge dates for this group were 27 or 28 October, 2003. This monkey was presented for necropsy on the day of death. A complete necropsy was performed but only select tissues were taken for histopathologic examination, according to instructions from the PI.

Pathological Summary

Gross Pathology:
This intact male cynomolgus appears in good flesh with only a small amount of bloody fluid in and around the nares. The bronchi and trachea are filled with pink (bloody) froth. The lungs are diffusely wet and heavy (edema) and have multifocal coalescing dark purple foci up to 5-6 cm diameter scattered throughout all lung lobes. There is only a very small amount of clear, reddish edema fluid in the chest cavity. No other gross lesions were noted.

Gross Diagnosis: Severe diffuse bronchopneumonia

The following organs were weighed at necropsy:

Organ weights (grams):
Thyroid gland: 0.8
Thymus: atrophic, not weighed
Lung (with bronchi): 148.5
Spleen: 19.1
Liver: 243.1
Adrenal glands L: 1.39 R: 1.4
Kidneys L: 23.5 R: 20.6
Brain: 91.3

Microscopic Diagnoses:
1. Tongue: Glossitis, chronic, superficial, multifocal, minimal.
2. Thyroid gland: Cyst, follicular, focal.
3. Trachea: Tracheitis, subacute, multifocal, mild, with occasional epithelial degeneration and necrosis.
4. Trachea: Tracheitis, chronic, multifocal, mild.
5. Lung, diaphragmatic and apical lobes: Pneumonia, subacute, necrotizing and suppurative, diffuse, severe with alveolar flooding, multifocal bronchiolar epithelial necrosis and loss, mild multifocal hemorrhage, diffuse vascular congestion, multifocal vascular necrosis, mild multifocal fibrinous pleuritis, and myriad intra-alveolar bacteria, etiology consistent with *Yersinia pestis*.
6. Tracheobronchial lymph node: Lymphadenitis, subacute, necrotizing and suppurative, diffuse, severe with numerous bacilli.
7. Bronchus: Bronchitis, subacute, diffuse, mild with epithelial necrosis and erosion and bacilli.
8. Liver: Hepatitis, subacute, multifocal, portal and random, minimal.

Pathological Summary Continued...

9. Spleen, red pulp: Congestion, diffuse, mild.
10. Kidneys: Vascular congestion and edema, diffuse, mild.
11. Adrenal glands: Vascular congestion and edema, diffuse, mild to moderate.
12. Mandibular lymph node: Draining mild subacute inflammation.
13. Mesenteric lymph node: Sinus histiocytosis, diffuse, mild with draining minimal subacute inflammation.
14. Urinary bladder; gallbladder; kidneys; thymus; brain: No significant lesions.

COMMENTS: The lesions observed in this animal are consistent with *Yersinia pestis* infection. The cause of death was severe necrosuppurative bronchopneumonia. The lungs were diffusely affected. Over 90% of small airways and alveoli contain a mixture of viable and degenerate neutrophils, necrotic debris, small numbers of macrophages and high numbers of extracellular bacilli. The remainder of the small airways and alveoli are filled with a variable mixture of edema fluid, leukocytes and few erythrocytes. Bacilli were frequently seen within alveolar macrophages. An overt septicemia was not observed in this case. The multifocal subacute hepatitis may have been caused by plague bacilli in the circulation. A mild chronic portal hepatitis was observed but was not coded above. Autolysis hampered the microscopic evaluation of the kidneys. The thymus had undergone normal physiologic atrophy and only tiny remnants remained; it was not weighed at necropsy.

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